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Introduction

Lung cancer is the most common cancer in the world regarding to both incidence and mortality. It causes about 1.4 million deaths every year, which corresponds to almost one fourth of total cancer related deaths (1). To better understand the molecular mechanism of lung cancer and explore new therapies, good animal models that can faithfully recapitulate human disease is greatly needed. Recent whole genome sequencing data revealed at least 26 genes that are highly mutated in human lung adenocarcinoma samples (2, 3). In the original application, we proposed to establish a novel mouse model of lung cancer using our prestige lentiviral technology. The advantage of lentiviral vector is its capability of infecting any cell types and integrating into the genomic DNA (4). Thus, lentiviral vectors can be used to deliver oncogenes and shRNAs to defined cell types and initiate tumors in physiological environment. Our mouse model allows us to study these cancer mutations without generate huge amount of knockout or transgenic mouse lines. We proposed to use this unique mouse lung cancer model to study the crosstalk of tumor suppressor pathways, the cell origin of lung adenocarcinoma and the possible link of somatic cell reprogramming and tumorigenesis. We will summarize the research work conducted over the entire one year, which was supported by the FY09 LCRP Concept Award.

Body

We have proposed to establish a novel mouse lung cancer model based on our lentiviral gene transfer technique, and to study the crosstalk of tumor suppressor pathways, the cell origin of lung adenocarcinoma and the possible link of somatic cell reprogramming and tumorigenesis. In the next paragraphs, we will describe our research accomplishments associated with each task outlined in the approved Statement of Work.

1. shRNA sequences verification.

Our research work started with the designing of shRNA sequences to knockdown a bunch of tumor suppressors that have been identified in the whole genome sequencing of lung cancer samples. So far, we have cloned and verified shRNAs against p53, p16/Ink4a, p19/Arf, LKB1, pTEN, NF1, GSK3, APC, ATM, TSC1/2 and DLC1. Most of the shRNA sequences are able to efficiently knockdown gene expression (>70% by RT-PCR and western blotting analysis) after infection of mouse fibroblast cells. Majority of the shRNAs have been further subcloned into our CA2Cre vector that is used to induce lung cancer in KrasG12D mice.

2. Designing of lentiviral vectors to induce tumor in vivo.

To pursue **Specific Aim 1**, we have designed lentiviral vectors with shRNA combinations. Our CA2Cre lentiviral vector (Fig. 1a) is used to activate KrasG12D mutation by Cre recombination in LSL-Kras mice developed in Jacks lab. As shown in Fig. 1b, we put one or two shRNAs in the CA2Cre vector to test the crosstalk between different tumor suppressor pathways. Lentiviral vectors we've made include CA2Cre, CA2Cre-shp53, CA2Cre-shp16, CA2Cre-shp19, CA2Cre-shLKB1, CA2Cre-shpTEN, CA2Cre-DLC1, CA2Cre-shp53-shp16, CA2Cre-shp53-shp19, CA2Cre-shp53-shLKB1, CA2Cre-shp53-shpTEN, CA2Cre-shLKB1-shp19, CA2Cre-shLKB1-shpTEN. To pursue **Specific Aim 2**, we have designed lentiviral vectors with different promoters, which include CA2Cre vs CC10Cre, CA2Cre-shp53-shp16 vs CC10Cre-shp53-shp16. To pursue **Specific Aim 3**, we have used a lentiviral vector which has four reprogramming factors (Myc, Oct4, Sox2, Klf4). All the vectors have been verified in mouse fibroblast cells (or 293T) for Cre expression and shRNA knockdown.

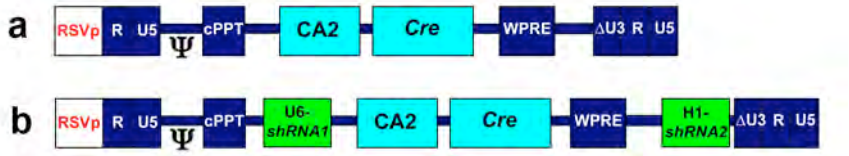

Figure 1. Diagram of lentiviral vector designing. Cre expression is controlled by tissue-specific promoter and shRNAs are driven by U6/H1 promoter. CA2, carbonic anhydrase 2 promoter.

3. Lentivirus production.

Lentivirus has been produced and concentrated according to the standard protocol (5). We used 6x15cm cell culture dishes to package each lentiviral vectors. The typical viral yield was 10^8 particles, which was further concentrated into smaller volume (100 ul). The final product for each vector was around 10^6/ul x100ul, which was sufficient for infecting 10-20 mice.

4. Animal model.

Mice strain: LSL-KrasG12D mice were bred and housed in Salk Animal Facility (SAF). 8-12 weeks old mice were used for lung cancer model via lentiviral infection. The KrasG12D allele was activated by the Cre recombination (Cre expression through lentiviral infection).

Infection procedure: mice were anesthetized with Ketamine/xylazine (i.p. 100 mg/kg). All the procedure was done in a clean and sterile area in the biosafety cabinet. All the surgical tools were autoclaved or sterilized. Under anesthesia, mouse tongue was pulled out with forceps and 20 ul of lentiviral suspension (10^5-10^6 viral particles) was delivered intra-tracheally using a lab pipette. After the infection, mice were constantly monitored in the cage on heating pad until full recovery from anesthesia. For the following 3 days, mice were monitored every day for any post-surgery infection, pain or distress. Mice infected with different lentiviral vectors were housed for 3-9 months to allow the development of tumors. Mice were collected at different time point to monitor the tumor progression.

Tumor development and mice survival: As shown in Fig. 2a, mice received CA2Cre-shp53 developed tumor much faster than those received CA2Cre. Three months after the lentiviral infection, mice started to show symptoms of carry lung cancer, including rapid breathing and being thin, hunched and scruffy. The median survival time for mice infected with CA2Cre-shp53 virus was 116 days (Fig. 2b).

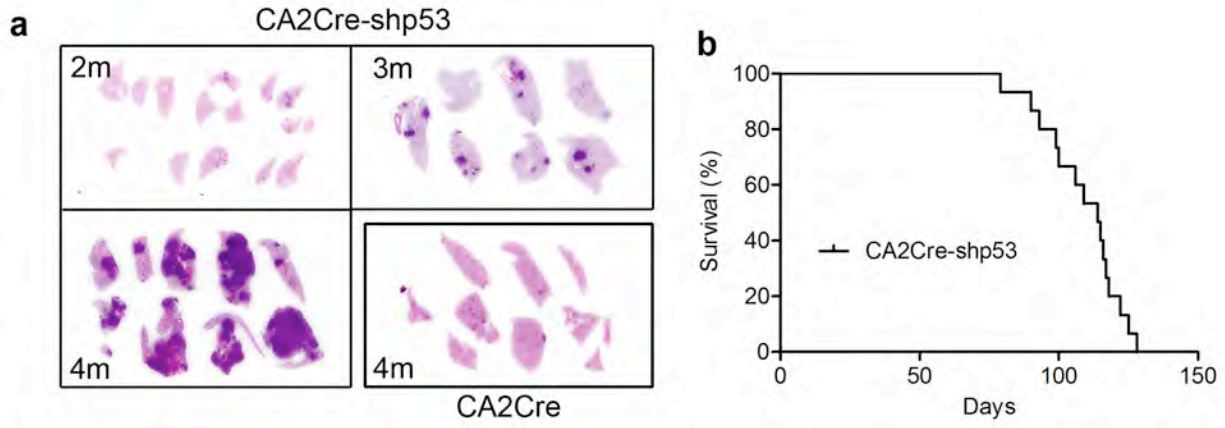

Figure 2. Kras^{G12D} mice were infected intra-tracheally with CA2Cre or CA2Cre-shp53 lentivirus. (a) Mice were harvested at 2, 3 or 4 months after the infection. Lung sections showed different tumour burdens. (b) Kaplan-Meier curve showing mice survival after CA2Cre-shp53 lentivirus infection.

5. Tumor sample analysis.

We have analyzed tumor samples collected from mice infected with different lentiviral vectors. These tumors were all adenomas and adenocarcinomas at different grade (6). Fig. 3a (i-iii) shows the evolution of tumor lesion from an atypical adenomatous hyperplasia (AAH), to small adenoma and to advanced adenocarcinoma. Twelve weeks after infection, majority of the tumor lesions reached Grade 3 and Grade 4, showing pleomorphic nuclei and aberrant mitosis (Fig. 3b). More importantly, massive stromal cell infiltration was found in ~20% of the advanced tumors, which recapitulates well the pathology observed in human malignancies (Fig. 3c). All tumors we have analyzed in CA2Cre-shp53 lentiviral model were typical adenocarcinomas with SPC (pro-surfactant protein C) positive and CC10 (Clara-cell specific antigen) negative staining patterns (Fig. 3d,e). The Ki-67 positive rate was 5-20% in these tumors, which is similar to what has been reported in human adenocarcinomas (Fig. 3f).

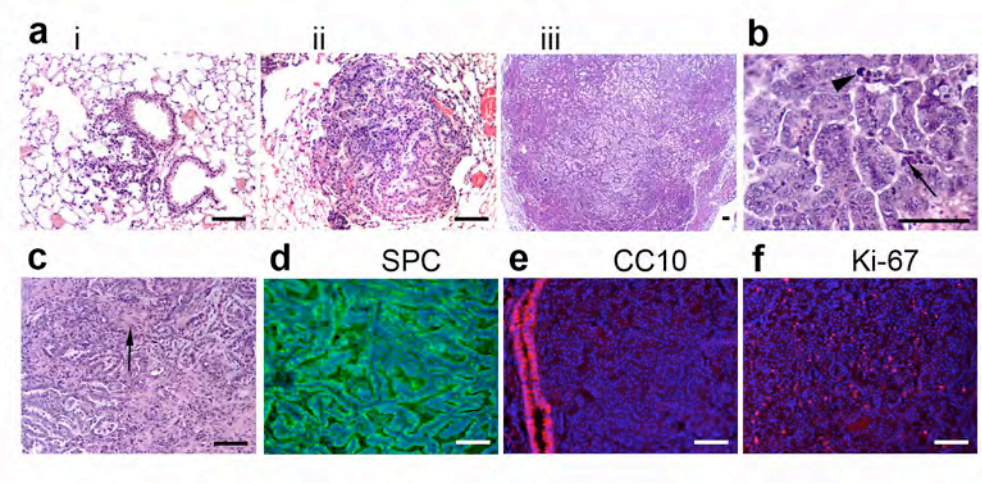

Figure 3. (a) Histology of atypical adenomatous hyperplasia (AAH, i), adenoma (ii) and adenocarcinoma (iii). (b) pleomorphic nuclei (arrow head) and aberrant mitosis (arrow) were found in advanced adenocarcinoma. (c) stromal cell infiltration. (d-f) tumor sections stained with SPC, CC10 and Ki-67 antibodies respectively.

In **Specific Aim 1**, we have proposed to study the cross-talk among tumor-suppressor pathways during tumorigenesis. Using our novel lentiviral vector system shown in Fig. 1b, we successfully bypassed the limitation of available conditional mouse lines and are able to study all the 26 mutations identified in the somatic mutation sequencing. So far, we have already tested KrasG12D mutation combined with shRNAs against p53, p16/Ink4a, LKB1, pTEN (Fig. 4a). We found that knocking down anyone of the tumor suppressor genes could significantly accelerate the tumor progression. We then started to test the crosstalk between p53 pathway and other important tumor suppressor pathways (Fig. 4b). P16/Ink4a-Cdk-Rb1 and p19/Arf-p53-p21 pathways control cell cycle checkpoint in parallel, and as we expected, combining shp53 and shp16 didn't significantly accelerate tumor development compared to shp53 alone. In contrast, pTEN is a potent inhibitor of Akt activation, so that shpTEN further increased the speed of tumor growth by activating Akt pathway. Interestingly, shLKB1 showed antagonistic effect to shp53, which was completely opposite to what we expected before. LKB1 controls AMPK and TSC1/2 tumor suppressor pathway, which limits mTOR activity and cell metabolism and proliferation. Consistent with what has been reported before (7), shLKB1 alone did show tumor promoting effect (Fig. 4a). More research work is undergoing to elucidate the possible mechanism how LKB1 and p53 pathways crosstalk with each other in the lung cancer development.

Besides, more lentiviral vectors containing shRNAs against NF1, GSK3, Rb1, APC, ATM, DLC1 and TSC1/2 are being tested. With these data, we will be able to study the synergistic and antagonistic effects between these pathways. All these findings will benefit the design of new lung cancer targeting therapies.

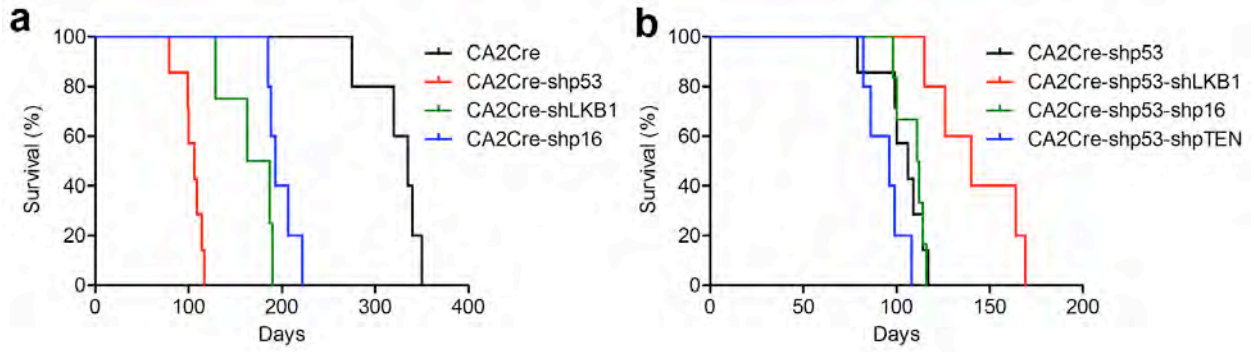

Figure 4. Kaplan-Meier curve of mice infected with indicated lentiviral vectors.

In **Specific Aim 2**, we have proposed to study of the cell origin of lung adenocarcinoma. The cell origin of lung cancer remains elusive (8). Taking the advantage of our lentiviral system, we used two different promoters to drive Cre expression and induced the lung tumorigenesis (Fig. 5a,b). CA2 (carbonic anhydrase 2) promoter is mainly active in type 2 alveolar cells in the distal lung, where adenocarcinomas are commonly found. CA2Cre vector successfully induced tumor initiation in the alveoli, and early lesions eventually developed into full-blown of adenocarcinomas similar to human tumors. CC10 (Clara cell 10kDa protein) promoter, instead, is active in all Clara cells and a limited number of type 2 alveolar cells (9). Interestingly, CC10Cre vector also gave rise to AAH and adenomas, which were identical to those induced by CA2Cre vector. Our data together indicated that lung adenocarcinomas are initiated from certain population of type 2 alveolar cells.

Furthermore, lentiviral vectors with combination of certain oncogenes and shRNAs against tumor suppressors have been made for investigating the cell origin of other forms of lung cancer, including squamous cell carcinoma (SCC), small cell lung carcinoma (SCLC) and large cell lung carcinoma (LCLC). It will be the unique tool to study these tumors in vivo, since there is no good model available for these lung cancers now.

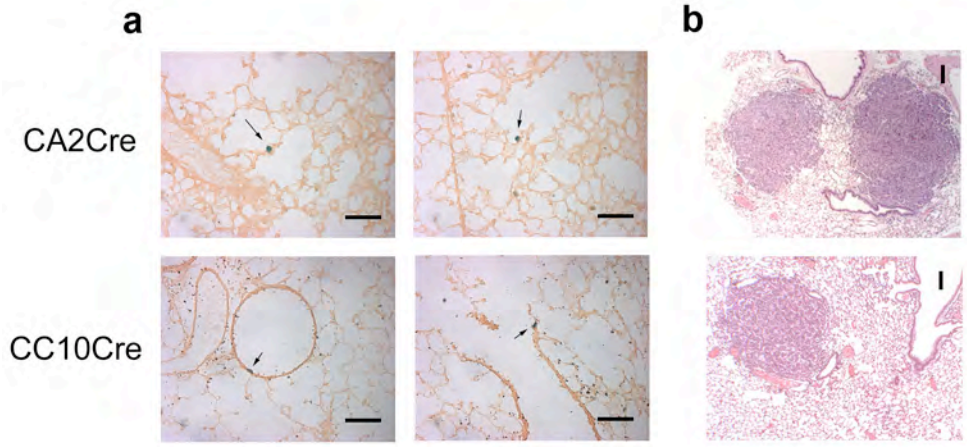

Figure 5. (a) Rosa26^{lacZ} mice were infected with CA2Cre or CC10Cre. β-galactosidase expression was examined by X-gal staining 14 days after the infection. Cells infected with lentiviruses and had Cre expression were stained blue (arrows). (b) Kras^{G12D} mice infected with CA2Cre or CC10Cre developed same adenomas.

In **Specific Aim 3**, we have proposed to compare reprogramming and tumorigenesis in parallel. The recent iPS studies indicated that some oncogenes (eg. Myc) and tumor suppressors (eg. p53) are playing important roles in regulating cell reprogramming. P53 knockdown substantially increases the reprogramming efficiency probably by interrupting cell cycle checkpoints. Interestingly, more than half of the cancer patients have acquired p53 mutations. Although the tumor suppressor function of p53 is at multiple levels, we can still make the hypothesis that certain step in malignant transformation is to mimic the reprogramming or dedifferentiation. Hence we used a lentiviral vector that expressed a combination of four reprogramming factors, including c-Myc, Oct4, Sox2 and Klf4. Till now (6 months after the infection), we haven't got solid tumors from those infected mice. Trouble-shooting is undergoing and possible problems may include: 1) The four factors induce cell apoptosis or senescence through p53 activation; 2) in vivo environment functions to suppress the reprogramming or transformation and induce re-differentiation. We are trying to use a new vector with four factors plus shp53 to see if this combination can improve the efficiency. These experiments will definitely help us to understand the tumor initiation, proliferation and differentiation and provide new concept for preventing and treating cancer.

Key Research Accomplishments

1. We have developed a mouse model of lung cancer by lentiviral gene delivery. Using shRNAs in the lentiviral vectors bypassed generating and crossing of mouse lines.
2. Knockdown of tumor suppressor genes by shRNAs accelerated tumor development. However, the crosstalk of tumor suppressor pathways led to synergistic or antagonistic effect in regulating tumor progression.
3. Our results suggested that lung adenocarcinomas are initiated in certain type 2 alveolar cells in vivo.
4. Reprogramming and tumorigenesis may share some common signal pathways, however the in vivo environment is prone differentiation, which may suppress tumorigenesis.

Reportable Outcomes

Meeting presentation:

Novel lung cancer model mediated by lentiviral gene delivery
Y. Xia, N. Yeddula, M. Leblanc, R.J. Shaw, I.M. Verma
2010. AACR-IASLC Joint Conference on Molecular Origins of Lung Cancer. San Diego CA.

Lentiviral Vector Mediated Lung Cancer Model.
Y. Xia, N. Yeddula, M. Leblanc, E. Ke, Y. Zhang, E. Oldfield, R.J. Shaw and I.M. Verma.
2011. Mechanism and models of Cancer (6th). La Jolla CA.

Manuscript:

Y. Xia, N. Yeddula, M. Leblanc, E. Ke, Y. Zhang, E. Oldfield, R.J. Shaw and I.M. Verma. Reduced cell proliferation by IKK2/IKKβ depletion in a mouse lung cancer model. (2011), Submitted.

Conclusion

Lentiviral vector is designed to deliver genes to almost any cell types. With tissue-specific promoter, we can control transgene expression in defined cell population. Taking the advantage of this unique tool, we developed a series of vectors to study the crosstalk of tumor suppressor pathways, the cell origin of lung cancer and the possible link of reprogramming and tumorigenesis. In the study of the crosstalk of tumor suppressor pathways, we cloned a set of shRNAs against the tumor suppressor genes mutated in human lung cancer and combined them with shRNA against p53. We found both synergistic and antagonistic effect between these shRNAs and p53 shRNA. This founding suggested the importance of considering the crosstalk between pathways when we design new targeted therapies. For example, activation of LKB1/AMPK pathway is beneficial to certain types of lung cancer, however, it may accelerate tumor progression in other cases which carry p53 mutations. The cell origin of lung cancer remains controversial. Our results from tissue specific promoters indicated that lung adenocarcinomas may be initiated from certain type 2 alveolar cells, which express both CA2 and CC10. The possible link between reprogramming, transformation and tumorigenesis attracts a lot of interest. These cellular events may share some common signal pathways, such as cell proliferation, escape from apoptosis, senescence and differentiation, and activation of 'stemness' pathways. We found that four reprogramming factors expression is not enough to transform somatic cell in vivo. Further identification of those factors or pathways that may suppress transformation will definitely benefit the research of tumor prevention. Altogether, our work supported by the LCRP Concept Award has established a new tool to study lung cancer in mouse. We will expand the study into other types of lung cancer including small cell lung cancer (SCLC), squamous cell lung cancer (SCC) and large cell lung cancer (LCLC). All these studies will immensely benefit the understanding of molecular mechanisms of lung cancer and provide new concept for designing new diagnostic tools and targeted therapies.

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